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(57) Abstract

The invention includes two classes of peptides and peptide derivatives that specifically inhibit the [TF:VIIa] initiated blood coagulation cascade. Class I peptides have the general formula $R'_1ZGHFGVR'_2$, whereas Class II peptides contain the core sequence -DHTGTKRS- and may be in either a linear or cyclic form. The invention encompasses the use of inhibitors as diagnostic reagents, as analytical reagents and as therapeutic drugs.

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PEPTIDYL INHIBITORS OF THE INITIATION OF COAGULATION

This application is a continuation-in-part of copending application serial no. 248,817, filed September 23, 1988.

BACKGROUND OF THE INVENTION

Cellular initiation of the coagulation protease cascades is usually attributable to the expression of tissue factor (TF) on the cell. TF is a transmembrane high affinity receptor (Ploplis et al. (1987) J. Biol. Chem. 262:9503; Morrissey et al. (1987) Cell 50:129) for factors VII or VIIa (its more active proteolytic derivative) (Zur, M. and Nemerson, Y. (1978) J. Biol Chem. 253:2203). The bimolecular complex of [TF:VII] or [TF:VIIa], both henceforth referred to as [TF:VIIa], function as a specific serine protease (Nemerson, Y. and Gentry, R. (1986) Biochem. 25:4020) that mediates activation of the zymogens factors X and IX to their active serine protease derivatives by limited proteolysis (Nemerson, Y. (1988) Blood 71:1). These mediate subsequent

steps of the extrinsic and intrinsic coagulation cascades, respectively.

Inhibition of the assembly of the [TF:VIIa] bimolecular complex by peptides based on the structure of tissue factor was first reported by Pepe, M. et al. (1988) Fed. Proc. 47, Abstract no. 552. Peptides corresponding to amino acids 26-49 and 146-167 of the sequence of tissue factor were reported to inhibit formation of the [TF:VIIa] complex and consequently to inhibit the activation of factor X. The complete deduced amino acid sequence of TF was first reported by Morrissey et al. (1987). In a related U.S. application, serial no. 178,495, peptides and peptide derivatives were disclosed having the ability to inhibit the proteolytic conversion of factor X to Xa by the activated [TF:VIIa] complex. The primary amino acid sequence of factor VII has also been deduced from the cloned nucleotide sequence encoding factor VII (O'Hara et al. (1987) Proc. Natl. Acad. Sci. USA 84:5158-5162). The amino acid sequence including the probable catalytic and binding sites were predicted from the nucleotide sequence (Hagan et al. (1986) Proc. Natl. Acad. Sci. 83:2412-2416). A tentative structure for human prepro Factor VII was postulated by Davie (1984) in Hemostasis and Thrombosis. Basic Principles and Clinical Practice, Second Edition, R. W. Colman et al. (eds.), Lippincott Co., Philadelphia, PA, pp. 242-267. Proposed disulfide bonds, inserted into the amino acid sequence of Factor VII by analogy to other established proproteins, indicated that the part of the amino acid

sequence containing the tissue factor binding site could in theory comprise a series of 8 loops.

The compounds of the invention are used as analytical reagents and therapeutic agents to specifically inhibit the initiation of the coagulation protease cascades by [TF:VIIa]. The compounds also permit accurate in vitro and ex vivo determination whether or not activation of coagulation is attributable to the binary complex [TF:VIIa]. The compounds are used as therapeutic drugs in vivo to inhibit the initiation of the coagulation system which is one of the pathogenetic mechanisms involved in thrombus formation and thrombotic related diseases, disseminated intravascular coagulation associated with septic shock and other disease processes, and certain inflammatory conditions associated with excessive activation of coagulation in the tissues.

SUMMARY OF THE INVENTION

The invention includes peptide and peptide derivatives that specifically inhibit blood clotting. The compounds of the invention were shown to inhibit the [TF:VIIa] initiated coagulation protease cascade leading to blood clot formation. Two different classes of peptides have been found to inhibit [TF:VIIa] initiated coagulation. All peptides are described by amino acid sequence, using standard abbreviations known in the art, either single letter abbreviations or three-letter abbreviations. In sequences using single-letter

abbreviations, the amino acids are understood to be linked by peptide bands between the α-amino group of one amino acid and the α-carboxyl of the adjacent amino acid. All amino acids described herein are L-amino acids unless specified otherwise.

DETAILED DESCRIPTION OF THE INVENTION

Class I peptides have the general formula

$$R'_1ZGHFGVR'_2$$

where R'_1 is hydrogen, an amino acid, derivatized amino acid, a peptide, a derivatized peptide, or a protein; R'_2 is hydroxyl, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide, or a protein; Z is Valine or Glycine. A derivatized amino acid or derivatized peptide is one wherein functional side or end groups are protected by reaction with a reactant to form an adduct or compound therewith. Many such amino acid derivatives, or peptide derivatives are known including, for example, amides, esters, t-butoxy carbonyls, carbobenzoxys, tosyls, benzyls, 7-amino 4-methyl coumarins and the like, all as well-known in the art. The sequence VGHFGV is found at position 372-377 in the amino acid sequence of factor VII.

Class II peptides have the general formula

$$R'_1SDHTGTKRSCR'_2$$

where R'_1 and R'_2 are defined as for class I peptides. The sequence SDHTGTKRSC is found at position 103-112 in the amino

acid sequence of factor VII. When R'_1 is cysteine, the resultant peptide specifies the amino acid sequence at position 102-112 of factor VII. This undecameric sequence spans the greater part of a loop structure within the native peptide structure of factor VII, as tentatively depicted by Davie (1984) in Hemostasis and Thrombosis. Basic Principles and Clinical Practice, Second Edition, R.W. Coleman et al. (eds.), Lippincott Co., Philadelphia, PA, p. 261. It is believed that several loops comprise this specialized domain within factor VII. Synthetic peptides having amino acid sequences derived from this region of factor VII can be prepared either as linear or cyclic peptides. Cyclization of the peptide is induced through formation of disulfide bonds.

It will be understood that modifications of the defined class I and class II peptides can be made, the modified peptides retaining activity as inhibitors of blood clotting. Such modifications include, for example, amino acid substitutions and utilization of different amino acid isomeric forms, e.g. D-amino acids. Not all substitutions or deletions would be expected to, nor do they yield inhibitors. However, those of ordinary skill in the art will recognize that certain substitutions are more likely to yield equivalent activity than others. Substituents with similar properties, for example, hydrophobic side chains, are more likely to yield active equivalents when replacing amino acids with similar side chains. Substitution of negatively charged for negatively charged, positively charged for positively charged,

aromatic for aromatic all have a higher likelihood of success than nonsimilar substitutions. The operating principles for choosing the most probable active equivalents are well-known in the art and active equivalents can be found according to the teachings herein without undue experimentation. Deletions can also yield active equivalents, particularly in the case of class II peptides. Truncated or internally deleted peptides comprising at least a portion of the class II sequence can be found without undue experimentation following the teachings of the invention. Further, peptides comprising tandem repeats of a desired sequence can be prepared according to the teachings of the present invention without undue experimentation.

Activity in blood clotting inhibition is measured by an assay for factor X activation. Purified factor X, when activated (converted to Xa) hydrolyses a chromogenic substrate S-2222 N-Benzoyl-L-Isoleucyl-L-Glutamyl-L-Arginine-p-Nitroanilide Hydrochloride (Helena Labs, Beaumont, Texas). Hydrolysis of S-2222 leads to a color change measurable by a change in absorbance at 405 nm. Activation of factor X depends in turn on formation of an active [TF:VIIa] complex. In the assay described herein factor VII was purified as described by Fair, D.S. (1983) Blood 62:784. Tissue factor (TF) was provided by a cell line, J82, derived from human bladder carcinoma, and publicly available from American Type Culture Collection, Rockville, MD, under accession No. ATCC HTB1. It has been previously demonstrated that factor VII and

factor VIIa bind to and are activated on the surface of J82 cells (Fair, D.S. et al. (1987) J. Biol. Chem. 262:11692. Factor X was purified as described by Schwartz, B.S. et al. (1981) J. Clin. Invest. 67:1650. In the assay system, purified factor VII or VIIa binds to TF forming an active [TF:VIIa] complex which in turn acts to convert factor X to Xa by specific proteolysis. Factor Xa then hydrolyzes S-2222, yielding a change in absorbance at 405 nm. The foregoing steps are considered the initial steps in the coagulation protease cascade that results in blood clot (thrombus) formation in whole blood. Inhibitors in the assay therefore act as clotting inhibitors.

Without being bound by any theory of mode of action of the inhibitors of the invention, class I and class II peptides are considered to inhibit the initial binding of TF with factor VII, preventing formation of an active [TF:VIIa] complex. Inhibition of S-2222 conversion in the assay is therefore considered to be the consequence of inhibition of the initial TF binding to factor VII. Inhibition is considered to be reversible and competitive for factor VII, either by directly interacting with TF or factor X or both.

Class I and class II peptides where R'_1 is hydrogen and R'_2 is hydroxyl, are not known to have any biological activity other than the activity disclosed. They are therefore specific inhibitors of [TF:VIIa] initiated blood coagulation. The possibility that other biological activity may be provided

by proper choice of R'_1 and/or R'_2 will be understood in the art. Multi-functional class I and class II peptides can be constructed by combining the core sequences with R'_1 and/or R'_2 peptides having known activity in themselves.

Peptides of class I and class II and pharmaceutically acceptable salts thereof are useful in the treatment of thrombosis, disseminated intravascular coagulation, septic shock and inflammation of cellular immune mediated diseases.

In practicing the present invention the compounds of class I and class II and pharmaceutically acceptable salts thereof may be used alone or mixed with a pharmaceutically acceptable carrier. Such compounds or salts can be administered to patients parenterally, for example subcutaneously, intravenously or intraperitoneally. Such compounds can be administered by intranasal instillation or by application to mucous membranes such as those of the sublingual region of the mouth or the nasal or bronchial mucosa as a spray, dry particle suspension or solution in water or saline solution.

EXAMPLES

Example 1: H-L-Ser-L-Asp-L-His-L-Thr-Gly-L-Thr-L-Lys-L-Arg-L-Ser-L-Cys.

Synthesis of this compound was performed on an Applied

Biosystems Model 430A peptide synthesizer using standard manufacturer's protocols for asymmetric anhydride formation for coupling to a PAM resin cartridge derivatized with N-Boc-S-4-Methylbenzyl-L-cysteine in which the first N-Boc-O-Bzl-L-Serine was coupled and followed by sequentially coupling with N-α-Boc-N-Tosyl-L-Arginine, N-α-Boc-N-ε- (2-ChloroCBZ)-L-Lysine, N-Boc-O-Bzl-L-Threonine, N-Boc-Glycine, N-Boc-O-Bzl-L-Threonine, N-α-Boc-N-im-Cbz-L-Histidine, N-Boc-L-Aspartic Acid-ß-Benzyl ester, and N-Boc-O-Bzl-L-Serine. The peptide was deprotected and cleaved from the PAM support by standard protocols and reagents including HF cleavage [anisole:resin:HF (1:1:10)] for 60 min at 0°C. Approximately 20-30 mg of product was purified on a Vydac C-18 column eluted with 10-40% (v/v) acetonitrile gradient, dried under vacuo. The product was used for analysis by dissolving it in water or desired aqueous solution.

Example 2: Gly-L-Ala-L-Thr-L-Val-Gly-L-His-L-Phe-Gly-L-Val-L-Tyr-L-Thr-L-Arg-L-Val-L-Ser-L-Gln-L-Tyr-L-Ile-L-Glu-L-Trp-L-Leu-L-Gln-L-Lys-L-Leu.

The synthesis was performed using the procedures in Example 1, except N-Boc-L-Leucine PAM resin was coupled with the following amino acid derivatives starting with N-α-Boc-N-ε-(2-ChloroCBZ)-L-Lysine and sequentially followed by N-α-Boc-L-Glutamine, N-Boc-L-Leucine, N-α-Boc-N-Indole-Formyl-L-Tryptophan, N-Boc-L-Glutamic acid-gamma-Benzyl ester, N-Boc-L-Isoleucine, N-Boc-O-(2-BromoCBZ)-L-Tyrosine, N-α-Boc-L-Glutamine, N-Boc-O-Bzl-Serine, N-Boc-L-Valine, N-α-Boc-N-

Tosyl-L-Arginine, N-Boc-O-Bzl-L-Threonine, N-Boc-O-(2-BromoCBZ)-L-Tyrosine, N-Boc-L-Valine, N-Boc-Glycine, N-Boc-L-Phenylalanine, N-α-Boc-N-im-Cbz-L-Histidine, N-Boc-Glycine, N-Boc-L-Valine, N-Boc-O-Bzl-L-Threonine, N-Boc-L-Alanine, N-Boc-Glycine. This peptide is deprotected and cleaved as stated in Example 1.

Example 3: Gly-Gly-L-His-L-Phe-Gly-L-Val-L-Tyr-L-Thr-L-Arg-Gly-Gly.

The synthesis was performed using the procedures in Example 1, N-Boc-Glycine Pam resin was coupled with N-Boc-Glycine and then sequentially with the following amino acid derivatives N-α-Boc-N-Tosyl-L-Arginine, N-Boc-O-Bzl-L-Threonine, N-Boc-O-(2-BromoCBZ)-L-Tyrosine, N-Boc-L-Valine, N-Boc-Glycine, N-Boc-L-Phenylalanine, N-α-Boc-Im-cbz-L-Histidine, N-Boc-Glycine, N-Boc-Glycine. The peptide was deblocked and cleaved as described in Example 1.

Example 4: L-Ala-L-Thr-L-Val-Gly-L-His-L-Phe-Gly-L-Val-L-Tyr-L-Thr-L-Arg-L-Val-L-Ser-L-Gln-L-Tyr-L-Ile-L-Glu-L-Trp-L-Leu-L-Gln-L-Lys-L-Leu-L-Met-L-Arg-L-Ser-L-Glu-L-Pro-L-Arg-L-Pro-Gly-L-Val-L-Leu-L-Leu-L-Arg-L-Ala-L-Pro-L-Phe-L-Pro-L-Cys.

The synthesis was performed as described in Example 1. N-Boc-S-4-Methylbenzyl-L-Cysteine PAM support resin was coupled with N-Boc-L-Proline and sequentially coupled with N-Boc-L-Phenylalanine, N-Boc-L-Proline, N-Boc-L-Alanine, N-α-Boc-N-Tosyl-L-Arginine, N-Boc-L-Leucine, N-Boc-L-Leucine, N-Boc-L-Valine, N-Boc-Glycine, N-Boc-L-Proline, N-α-Boc-N-Tosyl-

L-Arginine, N-Boc-L-Proline, N-Boc-L-Glutamic acid-gamma-Benzyl ester, N-Boc-O-Bzl-L-Serine, N-α-Boc-N-Tosyl-L-Arginine, N-Boc-L-Methionine, N-Boc-L-Leucine, N-α-Boc-N-ε-(2-ChloroCBZ)-L-Lysine, N-α-Boc-L-Glutamine, N-Boc-L-Leucine, N-α-Boc-N-Indole-Formyl-L-Tryptophan, N-Boc-L-Glutamic acid-gamma-Benzyl ester, N-Boc-L-Isoleucine, N-Boc-O-(2-BromoCBZ)-L-Tyrosine, N-α-Boc-L-Glutamine, N-Boc-O-Bzl-L-Serine, N-Boc-L-Valine, N-α-Boc-N-Tosyl-L-Arginine, N-Boc-O-Bzl-L-Threonine, N-Boc-O-(2-BromoCBZ)-L-Tyrosine, N-Boc-L-Valine, N-Boc-Glycine, N-Boc-L-Phenylalanine, N-α-Boc-N-im-Cbz-L-Histidine, N-Boc-Glycine, N-Boc-L-Valine, N-Boc-O-Bzl-L-Threonine, N-Boc-L-Alanine. The peptide was deprotected and cleaved as described in Example 1.

Example 5: Gly-L-Cys-L-Ser-L-Asp-L-His-L-Thr-Gly-L-Thr-L-Lys-L-Arg-L-Ser-L-Cys-Gly

(a) Linear peptide

The synthesis was performed using the procedures in Example 1, except that N-Boc-Glycine PAM resin was coupled sequentially with the following amino acid derivatives starting with N-Boc-S-acetamidomethyl-L-cysteine, N-Boc-O-Bzl-L-Serine, N-α-Boc-N-Tosyl-L-Arginine, N-α-Boc-N-ε-(2-ChloroCBZ)-L-Lysine, N-Boc-O-Bzl-L-Threonine, N-Boc-Glycine, O-Boc-O-Bzl-L-Threonine, N-α-Boc-N-im-Cbz-L-Histidine, N-Boc-L-Aspartic Acid-ß-Benzyl ester, N-Boc-O-Bzl-L-Serine, N-Boc-S-acetamidomethyl-L-cysteine, and L-Boc-Glycine. Coupling was

achieved with 1,3-dicyclohexyl carbodiimide in the presence of 1-hydroxybenzotriazole and cleaved with HF as described in Example 1.

(b) Cyclic Peptide

The linear peptide (synthesized as described in Example 5(a)) was cyclized through disulfide bond formation. The linear peptide (0.05 mmol) was dissolved in 350 μl of methanol:water (1:6) at room temperature and the solution was stirred while adding 50 μl of 1mM iodine in methanol dropwise for one hour at 4°C. The solution was stirred at 4°C for 2 days. The reaction was completely quenched with 1M sodium thiosulfate, lyophilized and then desalted by HPLC. The cyclization procedure is detailed by Stewart and Young (1984) in Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL.

Example 6: L-Cys-L-Glu-L-Gln-L-Ile-L-Ser-L-Ser-L-Asp-L-His-L-Thr-Gly-L-Thr-L-Lys-L-Arg-L-Ser-L-Cys

(a) Linear Peptide

The synthesis was performed using the procedures in Example 1, except that N-Boc-S-acetamidomethyl-L-cysteine PAM resin was coupled sequentially with the folloing amino acid derivatives starting with N-Boc-O-Bzl-L-Serine, N-α-Boc-N-Tosyl-L-Arginine, N-α-Boc-N-ε-(2-ChloroCBZ)-L-Lysine, N-Boc-

O-Bzl-L-Threonine, N-Boc-Glycine, N-Boc-O-Bzl-L-Threonine,N-α-Boc-N-im-Cbz-L-Histidine, N-Boc-L-Aspartic Acid-ß-Benzyl ester, N-Boc-O-Bzl-L-Serine, N-Boc-O-Bzl-L-Serine, N-Boc-L-Isoleucine, N-α-Boc-L-Glutamine, N-Boc-L-Glutamic acid-gamma-Benzyl ester, and N-Boc-S-acetamidomethyl-L-cysteine. Coupling was achieved with 1,3-dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole and cleaved with HF as described in Example 1.

(b) Cyclic peptide

The linear peptide (synthesized as described in Example 6(a)) was cyclized through disulfide bond formation essentially as described in Example 5(b).

Example 7: Inhibition Studies Using Peptides Derived From the Core Sequence of Class I and II Inhibitors

Various peptides, including those synthesized essentially as described in Examples 1-6, were tested for inhibitor activity. Using the assay system described in the legend of Table 1, the peptides listed therein were assayed for inhibitory potency at concentrations up to 1mM. From the results of inhibitory activity of the peptides of Examples 2-4 (Class I peptides), a series of overlapping hexapeptides within the sequence TVGHFGVYTRV was synthesized essentially as described in Example 1. The peptides were assayed for inhibitory activity with results shown in Table 2. Maximal inhibitory activity was observed in peptides having a core

TABLE 1

Relative Efficacy of Tested Peptides

Amino Acid Sequence	Relative Potency (Ki5, MU)	Inhibitor Class
CASSC	0	
CQNGGSC	0	
CKDQLQSYIC	0	
KDQLQSY	0	
CLPAFEGRNC	0	
CETHKDDQ	0	
CETHKDDQLIC	0	
CVNENGGCEQYC	0	
SDHTGTKRSC	25	II
CHEGYSLLADGVSC	0	
CTPTVEYPC	0	
CGKIPILEKRNASKPQGRG	0	
GEHDLSEHDGDEQSRRVAQ-GC	0	
PERTFSERT-GC	0	
PRLMTQDC	0	
CLQQSRKVGDSPNITEYMFC	0	
G-ATVGHFGVYTRVSQYIEWLQKL-G	45	I
G-GHFGVYTR-GG	49	I
ATVGHFGVYTRVSQYIEWLQKLMRS-EPRPGVLLRAPFP-C	52	I
IEWLQHLMRSEPRPGVLLRAPFP-C	0	

Relative potency is expressed as megaunits (MU), where one unit is equal to the inverse of the molar concentration at 5% inhibition of clotting as assessed by inhibition of factor X activation. Quantitation of inhibition was performed in a linked enzyme chromogenic assay using purified factor VII (Fair, 1983) and factor X (Schwartz et al., 1981), TF positive cells (J82) and chromogenic substrate S-2222 (Helena Labs, Beaumont, Texas). The peptides were simultaneously incubated with 1 nM factor VII, 100 nM factor X, 20mM CaCl2, 1X105 J82 cells (American Type Culture Collection, Rockville, MD, under accession no. ATCC HTB1) and 2 mM S-2222 in a total volume of 225 uL. The rate of conversion of factor X to Xa was monitored kinetically by the change in absorbance of the chromogenic product of S-2222 at 405 nm.

TABLE 2

Inhibition of factor X Activation by Peptides Derived from the TVGHFGVYTRV Peptide Sequence of Class I Inhibitor

Amino Acid Sequence	Relative Potency (Ki5, MU)
TVGHFG	10
VGHFGV	45
GHFGVY	0
HFGVYT	0
FGVYTR	0
GVYTRV	0

Peptides which were 6 amino acids in length were synthesized based upon the amino acid sequences of peptides of Examples 2-4 which inhibit [TF:VIIa]-initiated activation of factor X (Table 1). The peptides were identified for synthesis by moving a 6 mer bracket along the amino acid sequence TVGHFGVYTRV. Activity was measured as described in Table 1.

sequence ZGHFGV, where Z is G (as in the peptide of Example 3) or V as in the peptides of Examples 2, 4 and Table 2, line 2. Peptides having the foregoing core plus additional amino acids, peptides or proteins at the amino or carboxyl ends are also active as inhibitors. Therefore, peptides of class I, R'_1ZGHFGVR'_2, where Z is V or G and R'_1 is hydrogen, an amino acid, derivatized amino acid, a peptide, a derivatized peptide or a protein, and R'_2 is OH, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein are inhibitors of the present invention.

Similarly, the sequence SDHTGTKRSC shown in Table 1 to be relatively efficacious as a class II inhibitor was tested for the presence of an obligatory core sequence for inhibitory potency of the class II type. Synthetic peptides were prepared essentially as described in Example 1 wherein the sequence under study was decreased by one amino acid in sequential fashion starting from the N-terminal as well as from the C-terminal end of the molecule. The peptides were assayed for inhibitory activity with results shown in Table 3. Inhibitory activity was observed in peptides having the eight-member core sequence DHTGTKRS, as indicated in Table 3 and Table 1. When this octapeptide was expanded to include serine at the N-terminal, inhibitory activity was enhanced. The further addition of cysteine at the C-terminal position, additionally increased the inhibitory activity of the peptide. Moreover, when cysteine was now added at the N-terminal position of this extended peptide, the resultant peptide

TABLE 3

Inhibition of Factor X Activation by Peptides Derived from the SDHTGTKRSC Peptide Sequence (Class II Inhibitor)

Amino Acid Sequence	Relative Potency (K_i5,MU)
SDHTGTKRSC	25
SDHTGTKRS	20
SDHTGTKR	0
SDHTGTK	0
SDHTGT	0
SDHTG	0
DHTGTKRS	13
DHTGTKR	0
DHTGT	0
HTGTKRS	0
HTGTKR	0
HTGTK	0
TGTKRS	0
TGTKR	0
GTKRS	0

Peptides were identified for synthesis by sequential removal of one nucleotide at each end of the peptide. Synthesis was performed as outlined in Example 1. Activity was measured as described in Table 1.

showed inhibitory activity. Therefore, the general formulae of peptides of class II can be shortened to include an octapeptidyl core, i.e., R'_1DHTGTKRSR'$_2$, where R'_1 is hydrogen, an amino acid, derivatized amino acid, a peptide, a derivatized peptide or a protein. and R'_2 is OH, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein, are inhibitors of the present invention. In a preferred embodiment of this invention, class II peptides had the shortened core formula R'_1DHTGTKRSR'$_2$ where R'_1 is L-serine and R'_2 is L-cysteine. Moreover, it was also preferred that the N-terminal amino acid be acetylated and the C-terminal amino acid be in the amide form.

The ten member core sequence shown to have inhibitory activity in Table 1 was contained within the peptide GCSDHTGTKRSCG (Table 4) which was synthesized both as a linear and a cyclized peptide. This peptide is a class II inhibitor, having the formula R'_1SDHTGTKRSCR'$_2$ described above where R'_1 is Gly-Cys and R'_2 is Gly. In the clotting assay assessed by inhibition of factor X activation, both the linear and cyclic forms of this peptide were biologically active. The activity of the cyclic form, which was cyclized by virtue of disulfide bond formation, was approximately 10 times greater than that of the linear form of the peptide.

Also, the ten-member core sequence -SDHTGTKRSC- was extended to include at the N-terminal end a pentapeptide and a cysteine at the carboxyl end of the molecule. The resultant

TABLE 4

Relative Efficacy of Linear and Cyclized GCSDHTGTKRSCG Peptide Class I Inhibitors

Amino Acid Sequence	Relative Potency (K_i5, MU)
GCSDHTGTKRSCG	25
GCSDHTGTKRSCG	233

Relative potency is expressed as megaunits (MU), where one unit is equal to the inverse of the molar concentration at 5% inhibition of clotting as assessed by inhibition of factor X activation. Quantitation of inhibition was performed in a linked enzyme chromogenic assay using purified factor VII (Fair (1983) Blood 62:784--791) and factor X (Schwartz et al. (1981) J. Clin. Invest. 67:1650-1658), TF positive cells (J82) and chromogenic substrate S-2222 (Helena Labs, Beaumont, Texas). The peptides were simultaneously incubated with 1 nM factor VII, 100 nM factor X, 20mM CaCl2, 1X105 J82 cells (American Type Culture Collection, Rockville, MD, under accession no. ATCC HTB1) and 2 mM S-2222 in a total volume of 225 μL. The rate of conversion of factor X to Xa was monitored kinetically by the change in absorbance of the chromogenic product of S-2222 at 405 nm.

peptide containing fifteen amino acids was synthesized in both a linear and cyclic form. Both the linear and cyclic forms of this peptide were biologically active in the clotting assay assessed by inhibition of factor X activation. Thus, a peptide having the formula R'_1SDHTGTKRSCR'$_2$ wherein R'_1 is the pentapeptide, L-Cys-L-Glu-L-Gln-L-Ile-L-Ser and R'_2 is OH, is another specific example of a class II inhibitor.

Other cyclic peptides having class II inhibitory activity are contemplated in this invention. Such cyclic peptides are patterned after the cyclic peptides exemplified in this invention and include peptides wherein the number of amino acids comprising the peptide are increased in order to effectively widen or lengthen the resultant loop or to add additional loops. Cyclic peptides of this type are class II inhibitors of coagulation.

In order to extend the plasma half life of these inhibitor compounds, the peptides were derivatized such that the functional groups of the terminal amino acids were protected through chemical linkage. In the preferred embodiment the N-terminal amino acid was acetylated and the C-terminal amino acid was converted into an amide.

Other class I and class II peptides can be synthesized essentially as described in Examples 1-6, or by other suitable techniques of peptide synthesis available to the art.

CLAIMS

1. A peptide having the structure

$R'_1ZGHFGVR'_2$

where Z is V or G, R'_1 is hydrogen, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein and R'_2 is hydroxyl, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein.

2. A peptide according to claim 1, selected from the group consisting of GATVGHFGVYTRVSQUIEWLQKLG, GGHFGVYTRGG, ATVGHFGVYTRVSQUIEWLQKLMRSEPRPGVLLRAPFPC and VGHFGV.

3. A peptide according to claim 1 where Z is V, R'_1 is GAT and R'_2 is YTRVSQUIEWLQKLG.

4. A peptide according to claim 1 where Z is G, R'_1 is hydrogen and R'_2 is YTRGG.

5. A peptide according to claim 1 where Z is V, R'_1 is AT and R'_2 is YTRVSQUIEWLQKLMRSEPRPGVLLRAPFPC.

6. A peptide according to claim 1 where Z is V, R'_1 is hydrogen and R'_2 is hydroxyl.

7. A peptide having the structure

$R'_1DHTGTKRSR'_2$

or portion thereof capable of inhibiting [TF:VIIa] initiated coagulation, where R'_1 is hydrogen, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein; and R'_2 is hydroxyl, an amino acid, a derivitized amino acid, a peptide, a derivatized peptide or protein.

8. A peptide according to claim 7 wherein R'_1 is hydrogen and R'_2 is hydroxyl.

9. A peptide having the structure

$$R'_1SDHTGTKRSCR'_2$$

or portion thereof capable of inhibiting [TF:VIIa] initiated coagulation, where R'_1 is hydrogen, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein and R'_2 is hydroxyl, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or protein.

10. A peptide according to claim 7 wherein R'_1 is hydrogen and R'_2 is hydroxyl.

11. A peptide according to claim 7 having the sequence

$$R'_1CSDHTGTKRSCR'_2$$

or portion thereof capable of inhibiting [TF:VIIa] initiated coagulation, where R'_1 is hydrogen, an amino

acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein and R'_2 is hydroxyl, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or protein.

12. A peptide according to claim 9 wherein the cysteines of said sequence are joined in disulfide linkage.

13. A peptide according to claim 9 wherein $R'_1 = R'_2$ and is selected from the group consisting of glycine and derivatized glycine.

14. A peptide according to claim 11 wherein the cysteines of said sequence are joined in disulfide linkage.

15. A peptide according to claim 7 wherein R'_1 is the pentapeptide, L-Cys-L-Glu-L-Gln-L-Ile-L-Ser, and R'_2 is OH.

16. A peptide according to claim 13 wherein the cysteines of said sequence are joined in disulfide linkage.

17. A composition having inhibitory activity against [TF:VIIa]-initiated coagulation comprising a peptide of class I or class II, said class I peptide having the structure R'_1ZGHFGVR'_2, said class II peptide having the structure R'_1DHTGTKRSR$'_2$ or portion thereof, where Z is G or V, R'_1 is hydrogen, an amino acid, a derivatized

amino acid, a peptide, a derivatized peptide or a protein and R'_2 is hydroxyl, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein, or pharmaceutically acceptable salts thereof.

18. A therapeutic composition comprising the composition of claim 9 and a pharmaceutically acceptable diluent.

19. A method of inhibiting [TF:VIIa]-initiated coagulation comprising administering to an animal or human patient an effective amount of a composition having inhibitory activity against [TF:VIIa]-initiated activation of factor X comprising a peptide of class I or class II, said class I peptide having the structure R'_1ZGHFGVR'_2, said class II peptide having the structure R'_1DHTGTKRSR$'_2$ or portion thereof, in linear or cyclic form where Z is G or V, R'_1 is hydrogen, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein and R'_2 is hydroxyl, an amino acid, a derivatized amino acid, a peptide, a derivatized peptide or a protein, or pharmaceutically acceptable salts thereof.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04140

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 7/00, 7/06; A61K 37/02

U.S. Cl: 530/328,324,350,384; 514/16, 12

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/328,324,350,384; 514/16,I 2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proc. Natl. Acad. Sci. USA, Vol. 83, April 1986, F. Hagen, "Characterization of cDNA coding for human factor VII", pages 2412-2416, see page 2414, Fig. 2.	1,7 a
Y	Biochemistry, Vol. 27, 1988, Thim, Amino acid sequence and post translational modifications of human factor VIIa from plasma and transfected baby hamster kidney cells", pages 7785-7793, see page 7789, Figure 3 and page 7791, col. 2.	1,7,17 and 19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 December 1989	12FEB 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/USA	T. D. Wessendorf

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(See Attachment).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. ~~Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers~~: 1 - 8, 10 and 15, species having the structure shown in claim 7 wherein R_1 is hydrogen and R_2 is hydroxyl, (SEE ATTACHMENT-2)

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Cell, Vol. 50, 03 July 1987, J. Morrissey, "Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade", pages 129-135, see the entire document.	1-8,10,15, 17, 18, 19
A	Proc. Natl. Acad. Sci. USA, Vol. 84, August 1987, O'Hara, "Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation", pages 5158-5162, see the entire document.	1-8,10,15, 17, 18, 19
A	Federation Proceedings, Vol. 47, 15 September 1988, Thrombosis and Thrombolysis (552-557), N. Paps, "Functional sites on human tissue factor", see col. 1, no. 552.	1-8,10,15, 17, 18, 19